Approaches to the Choice of new Biomarkers in Research with Emphasis on Atherosclerosis

Professor Daniel Petrovič MD PhD, FESC

Institute of Histology and Embryology
Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia
International Center for Cardiovascular Diseases Medicor, Izola, Slovenia

dp.petrovic@gmail.com
daniel.petrovic@mf.uni-lj.si
GENE BIOLOGY AND BIOMARKERS

• **Increased knowledge of genes biology** is generating promising marker candidates for more accurate **diagnosis, prognosis assessment, and therapeutic targeting**.

• **Well-designed clinical trials** for assessing the utility of markers are needed.

• Ideally, the **population studied** should be one:
  – in which knowledge of the marker would have substantial clinical relevance and
  – where the feasibility of obtaining appropriate specimens is established.
COMPLEX TRAITS AND DEVELOPMENT OF NEW BIOMARKERS

- **Complex disorders** such as **atherosclerosis** (CAD, carotid disease), **diabetes** with micro- /macrovascular complications $\rightarrow$ **caused by** multiple genetic and environmental factors.

- **Difficulties in developing biomarkers** for complex traits are:
  - phenotypic heterogeneity of disorder
  - understanding the role of the **genetic factors** in the pathophysiology of the disease
  - Differences in **environmental factors** (in adulthood and during early human development) $\rightarrow$ **epigenetic effect**
  - Interaction among factors involved in the development of disorder

- The **identification of candidate genes** influencing **complex traits**
  - may help **predict the individuals who are predisposed** to disease
  - may lead to **new drug targets**
COMPLEX DISORDERS AND DEVELOPMENT OF GENOMIC BIOMARKERS

• Phenotypic and etiologic heterogeneity of complex disorders influences the ability:
  • to discover a biomarker and
  • to prove the clinical utility of the biomarker once identified.

• Very importantly, a specific polymorphism may have utility in one but not in another population → studies in a variety of populations are needed
2 MAIN TYPES OF GENETIC STUDIES WITH REGARD TO MARKER IDENTIFICATION

• CANDIDATE GENE APPROACH
  – we choose potential candidate genes and select potential genetic markers (SNPs) according to “a priori” hypothesis → gene/protein may or may not be confirmed as biomarker for disorder → test whether some polymorphism is functional (increased expression in blood, tissue)

• GENOME WIDE ASSOCIATION STUDIES
  – Scanning of the whole genome without “a priori” hypothesis → test whether some polymorphism is functional (increased expression in blood, tissue)
GWAS AND CAD

- GWAS studies have identified **58 independent loci** associated with CAD **contributing 13.3% to CAD heritability**.

- Most identified loci have **low allele frequency (<5%)** with minor contributions to CAD development.

- Their **exact function** → is known only for some of them → and is related to **inflammatory response, oxidative stress regulation, lipid function, transportation, endothelial dysfunction** and other pathogenic processes involved in atherosclerosis.

NEW BIOMARKERS AND ATHEROSCLEROSIS

Some new promising **serologic and genetic biomarkers** that provide significant **diagnostic and prognostic information** about **cardiovascular risk prediction and atherosclerosis** have so far been reported.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Predictive ability</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-sensitivity C-reactive protein</td>
<td>↑risk for CV events and mortality</td>
<td>[40,41]</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>↑risk of premature atherosclerosis</td>
<td>[42,43]</td>
</tr>
<tr>
<td>Apolipoprotein-associated phospholipase A2</td>
<td>correlates with the coronary HD and its severity</td>
<td>[44–46]</td>
</tr>
<tr>
<td>Matrix metalloproteinases</td>
<td>markers of plaque vulnerability and subclinical atherosclerosis, predictors of CVD and mortality</td>
<td>[47–51]</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>early detection of subclinical CAD, its severity, diagnosis of MI</td>
<td>[52,53]</td>
</tr>
<tr>
<td>Endothelin-1</td>
<td>correlates with increased CAD and ACS risk and severity</td>
<td>[54–56]</td>
</tr>
<tr>
<td>Natriuretic peptides</td>
<td>↑risk for CV events and mortality</td>
<td>[57,58]</td>
</tr>
<tr>
<td>High-sensitivity assays for cardiac troponin</td>
<td>predictor of HF, mortality, and incident coronary HD</td>
<td>[59–62]</td>
</tr>
<tr>
<td>Pregnancy-associated plasma protein-A</td>
<td>marker of plaque vulnerability and predictor of CVD and mortality</td>
<td>[63]</td>
</tr>
<tr>
<td>Growth differentiation factor 15</td>
<td>predictor of CV and all-cause mortality, unstable AP</td>
<td>[64,65]</td>
</tr>
<tr>
<td>Micro-RNAs</td>
<td>association with the acute MI, predictor of atherosclerosis</td>
<td>[25,37]</td>
</tr>
</tbody>
</table>

Abbreviations: ACS, acute coronary syndrome; AP, angina pectoris; CAD, coronary artery disease; CV, cardiovascular; CVD, cardiovascular disease; HD, heart disease; HF, heart failure; MI, myocardial infarction.

2 MAIN TYPES OF GENETIC STUDIES WITH REGARD TO MARKER IDENTIFICATION

- **CANDIDATE GENE APPROACH**
  - we choose potential candidate genes and select potential genetic markers (SNPs) according to "a priori" hypothesis → gene/protein may or may not be confirmed as biomarker for disorder → test whether some polymorphism is functional (increased expression in blood, tissue) **MODEL 1 PROSTATIC CANCER**

- **GENOME WIDE ASSOCIATION STUDIES**
  - Scanning of the whole genome without "a priori" hypothesis → test whether some polymorphism is functional (increased expression in blood, tissue)
In prostate cancer - the prostate-specific antigen (PSA) changes is an accepted pre- clinical biomarker → far from perfect in terms of specificity and sensitivity.

Prostate biopsies are invasive, must be performed repeatedly, and only sample a fraction of the prostate.

Several new potential GBs with potentially better specificity and sensitivity than PSA have been discovered and published.

- These include specific gene mutations (e.g., HPC1, RNase-L) and combination of SNPs
An increasing number of clinical studies are employing gene expression profiling PBMCs for the identification of novel transcriptional biomarkers of disease and markers predictive of clinical outcomes.
PROSTATIC CANCER AND POTENTIAL BIOMARKERS

- Oligoarray ("AndroChip 2") containing 190 genes → was selected on the basis of their proved or potential role in prostate cancerogenesis related to androgen signalling.

- Multiple genes were identified exhibiting differential expression
  - in androgen-dependent and androgen-independent cells.
  - during drug treatment.

- All genes on the “Androchip 2” show a detectable expression levels in peripheral blood cells (PBMC).
PROSTATIC CANCER AND POTENTIAL NEW BIOMARKERS

Classification of prostate cancer using a protease activity nanosensor library.

Proteases are an important class of enzymes that play a role in every hallmark of cancer; their activities could be leveraged as biomarkers.

- Panel of prostate cancer proteases through transcriptomic and proteomic analysis.

- This activity-based nanosensor library could be useful throughout clinical management of prostate cancer, with both diagnostic and prognostic utility.

2 MAIN TYPES OF GENETIC STUDIES WITH REGARD TO MARKERS IDENTIFICATION

- **CANDIDATE GENE APPROACH**
  - we choose potential candidate genes and select potential genetic markers (SNPs) according to "a priori" hypothesis → gene/protein may or may not be confirmed as biomarker for disorder → test whether some polymorphism is functional (increased expression in blood, tissue) **MODEL 2 DIABETIC RETINOPATHY**

- **GENOME WIDE ASSOCIATION STUDIES**
  - Scanning of the whole genome without "a priori" hypothesis → test whether some polymorphism is functional (increased expression in blood, tissue)
The aim of this study was to examine the role of the rs6060566 polymorphism of the reactive oxygen species modulator 1 (Romo-1) gene in the development of diabetic retinopathy (DR) in Caucasians with type 2 diabetes (T2DM).

**METHODS:**

A total of 806 subjects with T2DM were enrolled in cross-sectional case-control study: 278 patients with DR and 528 subjects without clinical signs of DR. Moreover, immunohistochemical analysis of 40 fibrovascular membranes of patients with proliferative DR was performed. The number of positive (labelled) cells per area - numerical areal density of the Romo-1-positive cells (the number of positive cells/mm(2) ) - was calculated.
DIABETIC RETINOPATHY AND POTENTIAL BIOMARKER ROMO-1

RESULTS:
A significantly higher frequency of the CC genotype of the rs6060566 polymorphism of the Romo-1 gene was found in subjects with T2DM with DR compared to those without DR (odds ratio=3.3, 95% confidence interval=1.1-8.8; p = 0.024).
Moreover, the Romo-1 C allele was found to effect Romo-1 expression in fibrovascular membranes of patients with proliferative DR.

CONCLUSIONS:
The rs6060566 polymorphism of the Romo-1 gene was found to be an independent risk factor for DR in Caucasians with T2DM. Moreover, the rs6060566 is most probably functional and its effect might be mediated through the increased expression of Romo-1 in the retina.
2 MAIN TYPES OF GENETIC STUDIES WITH REGARD TO MARKERS IDENTIFICATION

• CANDIDATE GENE APPROACH
  – We choose potential candidate genes and select potential genetic markers (SNPs) according to “a priori” hypothesis → gene/protein may or may not be confirmed as biomarker for disorder → test whether some polymorphism is functional (increased expression in blood, tissue) MODEL 3 – CAROTID Atherosclerosis

• GENOME WIDE ASSOCIATION STUDIES
  – Scanning of the whole genome without “a priori” hypothesis → test whether some polymorphism is functional (increased expression in blood, tissue)
SUBJECTS WITH T2DM AND CAROTID ATHEROSCLEROSIS – SLOVENIAN STUDY

- The design of the study was cross-sectional with follow-up for up to 4 years
- consecutive patients with type 2 diabetes from different General hospitals in Slovenia were enrolled

Inclusion criteria for cases:
- Caucasians above >50 years
  &
- with DM 2

Exclusion criteria:
- Evident CAD (history of myocardial infarction)
  &
- CV stroke

DNA extraction
Combine sample with TaqMan SNP Genotyping Assay
Run on real-time PCR System
Analyze data
Patients examination

- Doppler examinations of carotid arteries
  - Morphological data
  - Functional data

CIMT
  - Cut-off > 75 percentile

Plaque type
  - No plaque (0)
  - Unstable plaque (1, 2, 3) + plaque thickness
  - Stable plaque (4, 5) + plaque thickness

Plaque score
  - Number of affected arteries (CCA, bifurcation, ICA)
    - (0, 1, 2)
    - (3, 4, 5, 6)

- CT angio of coronary arteries
  - Coronary calcium score

ACI = 1
BIFF = 1
ACC = 1
1000 subjects are enrolled

700 cases
Doppler exams are available

300 cases
Doppler exams - are ongoing (2018)
WE EVALUATED GENE POLYMORPHISMS OF DIFFERENT PATHOGENETIC SYSTEMS (OXIDATIVE STRESS, INFLAMMATORY, GROWTH FACTORS etc.)

...as genetic markers of carotid atherosclerosis in type 2 DM

We wanted to learn if there are any differences in CIMT and presence of carotid plaques according to genotypes (risk genotypes vs. non-risk genotypes).


<table>
<thead>
<tr>
<th></th>
<th>Subjects with T2DM</th>
<th>Control group</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>62.39 ± 9.61</td>
<td>60.07 ± 9.18</td>
<td>0.008</td>
</tr>
<tr>
<td>Male sex (%)</td>
<td>338 (56.8)</td>
<td>92 (46.0)</td>
<td>0.008</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>11.25 ± 7.88</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cigarette smoking (%)</td>
<td>53 (8.91)</td>
<td>34 (17.0)</td>
<td>0.002</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>108.65 ± 12.88</td>
<td>93.31 ± 13.18</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>31.00 ± 4.74</td>
<td>27.90 ± 4.42</td>
<td>0.16</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>147.1 ± 19.80</td>
<td>143.3 ± 16.6</td>
<td>0.86</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>85.78 ± 11.60</td>
<td>84.7 ± 11.6</td>
<td>0.19</td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)</td>
<td>8.04 ± 2.57</td>
<td>5.27 ± 0.87</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.89 ± 3.56</td>
<td>4.79 ± 0.29</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.70 ± 1.18</td>
<td>5.36 ± 1.08</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.20 ± 0.35</td>
<td>1.43 ± 0.37</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>2.63 ± 0.94</td>
<td>3.24 ± 0.98</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.9 (1.2–2.7)</td>
<td>1.3 (0.9–1.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>hs-CRP (mg/L)</td>
<td>3.5 ± 1.18</td>
<td>2.2 ± 1.18</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CIMT (μm)</td>
<td>958 ± 194</td>
<td>890 ± 212</td>
<td>0.007</td>
</tr>
<tr>
<td>Statin therapy (%)</td>
<td>375 (63.0)</td>
<td>62 (31.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Antihypertensive agents (%)</td>
<td>499 (83.9)</td>
<td>58 (29%)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Continuous variables were expressed as means ± standard deviations when normally distributed and as median (interquartile range) when asymmetrical distributed. Categorical variables were expressed as frequency (percentage). BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; HbA1c: glycated haemoglobin; hs-CRP: high sensitivity C-reactive protein.
We found an association between the rs2071559 (KDR) and either CIMT or sum of plaque thickness in subjects with T2DM.
Vascular endothelial growth factor (VEGF) - rs2010963
Receptor for VEGF (KDR) - rs2071559

TABLE 2: VEGF serum levels in subjects with and without T2DM with regard to the rs2010963 and rs2071559 genotypes.

<table>
<thead>
<tr>
<th>rs2010963</th>
<th>Mean (95% CI)</th>
<th>p</th>
<th>Linear trend analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC (52)</td>
<td>VEGF (ng/L) 63.5 ± 29.2</td>
<td>&lt;0.01</td>
<td>3.22</td>
</tr>
<tr>
<td></td>
<td>CG + GG (543) 46.1 ± 22.3</td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>rs2071559</td>
<td>Mean (95% CI)</td>
<td></td>
<td>Linear trend analysis</td>
</tr>
<tr>
<td>CC (131)</td>
<td>VEGF (ng/L) 69.4 ± 25.1</td>
<td>&lt;0.01</td>
<td>3.70</td>
</tr>
<tr>
<td></td>
<td>CT + TT (464) 40.9 ± 28.3</td>
<td></td>
<td>0.02</td>
</tr>
</tbody>
</table>

Higher serum levels of VEGF were found in subjects with the CC genotypes of both polymorphisms (rs2010963, rs2071559) in comparison with subjects with other genotypes.

Increased expression of VEGF receptor was found in atherosclerotic plaques (endarterectomy sequester).
### Polymorphisms of the PPAR-γ - rs1801282

**Table 5: Association of the rs1801282 genotypes with the presence of plaques and presence of unstable plaques in patients with T2DM at the time of recruitment.**

<table>
<thead>
<tr>
<th>rs1801282</th>
<th>Presence of plaque</th>
<th>Presence of unstable plaque</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>p</td>
</tr>
<tr>
<td>Hypertension (0 = no; 1 = yes)</td>
<td>1.71 (0.93–2.58)</td>
<td><strong>0.04</strong></td>
</tr>
<tr>
<td>Systolic blood pressure (mm Hg)</td>
<td>1.07 (0.92–1.007)</td>
<td>0.17</td>
</tr>
<tr>
<td>LDL cholesterol (mmol/L)</td>
<td>1.21 (0.78–1.89)</td>
<td>0.40</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>0.18 (0.05–0.63)</td>
<td><strong>0.008</strong></td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.28 (0.63–1.03)</td>
<td>0.09</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>1.14 (0.64–1.54)</td>
<td>0.28</td>
</tr>
<tr>
<td>GC + GG*</td>
<td>0.79 (0.48–1.14)</td>
<td><strong>0.04</strong></td>
</tr>
</tbody>
</table>

---

**Polymorphisms of the PPAR-γ (rs1801282) and Its Coactivator (rs8192673) Have a Minor Effect on Markers of Carotid Atherosclerosis in Patients with Type 2 Diabetes Mellitus.**

Plesković A, Šantić Letonja M, Cokan Vujkovac A, Starčević JN, Petrović D.


PMID: 26949382  Free PMC Article
In our study, we found an association between the \textbf{rs12762303} and \textbf{coronary calcium score} in subjects with T2DM.
Moreover, we found an association between the \textbf{rs3802278} and CIMT in subjects with T2DM.
ECHO markers of subclinical carotid atherosclerosis in T2DM ASSOCIATION

**CIMT**
- rs2071559 KDR
- rs4646994 ACE
- rs3802278 ALOX5AP
- rs275651, rs931490 AT1R
- MMP3 (rs3025058)

**SUM OF PLAQUE THICKNESS**
- rs2071559 KDR
- rs4646994 ACE
- rs699 AGT
- IL-1α (rs1800587)
- IL-1β (rs1143634)
- PPARγ (rs1801282)
- SPP1 (rs4754)
- OPG (rs2073618)

**PLAQUE PROGRESSION**
- IL-1β (rs1143634)
- PPARGC1A (rs8192678)
ECHO markers of subclinical carotid atherosclerosis – NO ASSOCIATION

CIMT
MMP3 (rs3025058)
IL-1α (rs1800587)
IL-1β (rs1143634)
PPARγ (rs1801282)
SPP1 (rs4754)
OPG (rs2073618)
AGT (rs47629, AGT1R (rs275561, rs931490 rs5182)
ALOX5 (ss12762303)

SUM OF PLAQUE THICKNESS
MMP3 (rs3025058)
IL-1α (rs1800587)
IL-1β (rs1143634)
PPARγ (rs1801282)
SPP1 (rs4754)
OPG (rs2073618)
AGT (rs47629, AGT1R (rs275561, rs931490 rs5182)
ALOX5 (ss12762303)

PLAQUE PROGRESSION
MMP3 (rs3025058)
IL-1α (rs1800587)
IL-1β (rs1143634)
PPARγ (rs1801282)
SPP1 (rs4754)
OPG (rs2073618)
AGT (rs47629, AGT1R (rs275561, rs931490 rs5182)
ALOX5 (ss12762303)

......
2.4.1.3 Epigenetics

Epigenetics studies the chemical changes in DNA that affect gene expression. Methylation of genes related to CV risk factors is associated with variation in CV risk factor levels, and lower DNA methylation levels are associated with an increased risk of CAD or stroke. No information exists, however, regarding the effect of epigenetic markers in improving CVD risk prediction beyond conventional risk factors. Thus, epigenetic screening of CVD is not recommended.
Glutathione S- transferases (M1, T1) null alleles and ischemic vascular disease

- In meta-analysis including almost 20,000 cases with IVD (ischemic heart disease or ischemic stroke) and almost 50,000 controls in general population, GST (M1, T1) deletion genotypes did not associate with risk of IVD or with markers of inflammation (Norskov et al., 2011).

  - In our study in diabetics, **smokers with GSTM1 null genotype (deletion) had increased CIMT** in comparison with smokers **without null genotype** (1.18 vs. 1.11 mm) - **epigenetic effect** (interaction between genetic and environmental factors)

  - **Smokers with GSTM1 null genotype** were shown to have a greater CIMT as well as a higher 2-year progression rate of CIMT in general population (De Waart. 2001).


Recently several gene polymorphisms of oxidative stress, inflammatory, growth factors and RAS genes have been reported to be associated with macrovascular complications (MI, carotid atherosclerosis) in pts with type 2 diabetes.


STUDENTS FROM ABROAD
via STUDENT ORGANISATION SLOMSIC or via CMEPIUS
http://www2.cmepius.si/en/index.html
STUDENTS FROM ABROAD
via STUDENT ORGANISATION SLOMSIC or via CMEPIUS
http://www2.cmepius.si/en/index.html

dp.petrovic@gmail.com
daniel.petrovic@mf.uni-lj.si
Gene - Editorial Board

Co Editors-in-Chief

A.J. van Wijnen, PhD
Dept. of Orthopedic Surgery, Dept. Biochemistry and Molecular Biology, Mayo Clinic, 200 First Street SW, Rochester, Minnesota, MN 55905, USA

T. Gojobori
King Abdullah University of Science and Technology (KAUST), Thuwal, Saudi Arabia

Executive Editors

S. Chua de Sousa Lopes
Leida Universitair Medisch Centrum (LUMC), 2300 RC, Leiden, Netherlands

A.M. Engel
Dept. of Epidemiology, Tulane Cancer Center, Tulane University, 1430 Tulane Avenue, New Orleans, Louisiana, LA 70112-1699, USA

J. Messing
Waksman Institute, Rutgers University, 190 Frelinghuysen Road, New Brunswick, New Jersey, NJ 08904, USA

J. Pratap
Department of Anatomy and Cell Biology, Rush University Medical Center, 1753 W. Harrison St. Chicago, Illinois, IL 60612, USA

A.P. Rooney
National Center for Agricultural Utilization Research, U.S. Department of Agriculture (USDA), Agricultural Research Service (ARS), 1845 N University St, Peoria, Illinois, IL 61604-3299, USA

A. Rynych
Dept. of Molecular Oncogenetics, Inst. of Molecular Biology and Genetics, Ukrainian National Academy of Sciences (NAS), 156 Zabolotnogo str., 03143, Kiev, Ukraine

C. Vieira-Heddi
Bule Grégoire Mendel, UMR CNRS 5588 - LBBE, Université Claude Bernard - Lyon 1, 43 bd du 11 novembre 1918, 69621, Villeurbanne, France

Review Editor

D. Petrović
University of Ljubljana, Ljubljana, Slovenia

Gene Wiki Editors

E.A. Golemis
Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA
Approaches to the Choice of new Biomarkers in Research with Emphasis on Atherosclerosis

Professor Daniel Petrovič MD PhD, FESC

Institute of Histology and Embryology
Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia
International Center for Cardiovascular Diseases Medicor, Izola, Slovenia

dp.petrovic@gmail.com
daniel.petrovic@mf.uni-lj.si